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PCT

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(54) Title: USE OF PLATELET FACTOR 4 TO INHIBIT OSTEOBLAST PROLIFERATION

(57) Abstract

The invention features the use of platelet factor 4 (PF4) for inhibiting proliferation of osteoblasts in a mammal in need of such inhibition. The method entails administering PF4. PF4 can be used to treat both diseases characterized by primary changes in osteoblastic cell funtion/activity (e.g., osteosarcoma) and diseases or systemic conditions affecting bone in which abnormal osteoblastic cell function/activity is a secondary effect (e.g., osteoporosis). In addition, PF4 may be used to treat diseases associated with localized changes in bone metabolism in which abnormal osteoblastic cell function/activity contributes to pathogenic bone changes, e.g., periodontal disease, rheumatoid arthritis and osteoarthritis, localized osteoporosis, mastocytosis, multiple myeloma, and bone metastases of various tumors.

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USE OF PLATELET FACTOR 4 TO INHIBIT OSTEOBLAST PROLIFERATION

Statement as to Federally Sponsored Research

5   This work was supported in part by NIH Grant No. DE09915 and the government has certain rights in the invention.

Background of the Invention

Platelet factor 4 (PF4) is a well-known protein
10 which has been completely sequenced (Deuel et al. *Proc. Natl. Acad. Sci. USA* 78:4585, 1981). It is a 70-residue secretable platelet protein with a molecular weight of approximately 7.8 Kd which is released during platelet aggregation.

15   PF4 possesses characteristic structural features of the pro-inflammatory proteins interleukin-8 and β-thromboglobulin.

PF4 has been reported to inhibit the growth of melanoma and colon carcinoma *in vivo*, but not *in vitro*
20 (Sharpe et al. (1990) *J. Natl. Cancer Inst.* 82:848-853; Maione et al. (1991) *Cancer Res.* 51:2077-2083).

It has been suggested that PF4 may inhibit growth of Kaposi sarcoma cells *in vitro* (Zucker et al. (1991) *Proc. Soc. Exp. Biol. Med.* 198:693-702; Miles et al.
25 (1991) [abstract] *VII International Conference on Aids* (1991) Florence, Italy).

Hiti-Harper et al. (*Science* 199:991, 1978) report that PF4 inhibits collagenase derived from cultured human skin or human granulocytes.

30   Horton et al. (*Biochim. Biophys. Acta* 630:459, 1980) report that PF4 inhibits parathyroid hormone-stimulated ^{45}Ca release from fetal rate bone *in vitro*.

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Summary of the Invention

In general, the invention features a method, or
the use of PF4, for inhibiting proliferation of
osteoblasts in a mammal in need of such inhibition. The
5 method entails administering PF4. In a preferred
embodiment, the mammal is a human patient. In even more
preferred embodiments, the human patient is suffering
from osteosarcoma, the patient is suffering from
ossifying fibroma, the patient is suffering from osteoid
10 osteoma, the patient is suffering from fibrous dysplasia,
and the patient is suffering from osteoporosis. The
invention also features the use of PF4 for the
manufacture of a medicament for inhibiting proliferation
of osteoblasts in a mammal in need of such inhibition.
15   PF4 can be used to treat both diseases
characterized by primary changes in osteoblastic cell
function/activity (e.g., ossifying fibroma and fibrous
dysplasia, osteoblastoma and osteoid osteoma, and
osteosarcoma) and diseases or systemic conditions
20 affecting bone in which abnormal osteoblastic cell
function/activity is a secondary effect (e.g.,
acromegaly, hypercalcemia, primary or secondary
hyperparathyroidism, hyperthyroidism, osteoporosis, or
Paget's disease of bone). In addition, PF4 may be used
25 to treat diseases associated with localized changes in
bone metabolism in which abnormal osteoblastic cell
function/activity contributes to pathogenic bone changes.
For example, PF4 can be used to treat periodontal disease
(localized, inflammation-induced bone loss), rheumatoid
30 arthritis and osteoarthritis (localized, inflammation-
induced bone loss) localized osteoporosis, mastocytosis,
multiple myeloma, and bone metastases of various tumors.
        Because of its inhibitory effect on osteoblastic
cell proliferation, PF4 can be used to treat bone
35 abnormalities associated with either undesired

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osteoblastic cell proliferation or undesired osteoblastic
cell function or activity.

Natural or recombinant PF4 may be used in the
method of the invention. In addition, biologically
5   active fragments of PF4 having the ability to inhibit
proliferation of osteoblasts may be used. Such
biologically active fragments may be identified using the
osteoblast proliferation assay described herein.

Other features and advantages of the invention
10 will be apparent from the following description of the
preferred embodiments thereof, and from the claims.

                    Brief Description of the Drawings

FIGS. 1A to 1C are a set of graphs illustrating
the effect of PF4 on the proliferation of G-292 cells
15 grown in medium supplemented with 0.1% BSA (Fig. 1A), 2%
fetal calf serum (Fig. 1B), or 0.1% BSA and 0.5 U/ml
human alpha thrombin (Fig. 1C). Results are expressed as
percentage of the maximum response under the culture
condition tested and represent the mean ±S.E. of the
20 mean. The number of experiments is given in parenthesis
above the specific points. In each experiment, points
were tested in quadruplicates. Recent thymidine
incorporation is plotted as a function of PF4 dose in
ng/ml.

25   FIGS. 2A to 2C are a set of graphs illustrating
the effect of PF4 on the proliferation of Saos-2 cells
grown in medium supplemented with 0.1% BSA (Fig. 2A), 2%
fetal calf serum (Fig. 2B), or 0.1% BSA and 0.5 U/ml
human alpha thrombin (Fig. 2C). Results are expressed as
30 percentage of the maximum response under the culture
condition tested and represent the mean ±S.E. of the
mean. The number of experiments is given in parenthesis
above the specific points. In each experiment, points
were tested in quadruplicates. Percent thymidine
35 incorporation is plotted as a function of PF4 dose in ng/ml.

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FIGS. 3A and 3B are a set of graphs illustrating
the effect of indomethacin on PF4-induced inhibition of
proliferation of Saos-2 cells (Fig. 3A) and G-292 cells
(Fig. 3B). Results are expressed as percentage of
5 control (C) and represent the mean ±S.E. of the mean from
two experiments. In each experiment, points were tested
in quadruplicates.

Description of the Preferred Embodiment(s)

The experiments described below demonstrate that
10 PF4 inhibits proliferation of two osteoblastic
osteosarcoma cell lines (Saos-2 and G-292) in a dose-
dependent manner. This inhibition was observed whether
the cells were grown in serum-free media or were
stimulated with either serum or thrombin. This direct
15 effect on a osteoblastic cell line indicates that PF4 may
be used to treat conditions associated with abnormal
osteoblast activity. Further, it is known that cytokine-
and hormone-stimulated resorption requires osteoblastic
cells as mediators of the resorptive signal, even though
20 osteoclasts are the terminal effector cells (Heersche
(1989) in "Metabolic bone disease: Cellular and tissue
mechanisms", *CRC Press* pp. 1-17) thus PF4 may be useful
for treatment of disease conditions associated with
inappropriate levels of osteoclast activity or
25 proliferation.

The direct effect of PF4 against the osteosarcoma
cells, the lack of PF4 toxic effects when given *in vivo*,
and the immunoregulatory activities of PF4, all suggest
that PF4 may be used to treat osteosarcoma *in vivo*.
30   The two human osteoblastic osteosarcoma cell lines
Saos-2 (American Type Culture Collection, Bethesda, MD;
CRL 1423) (Rodan et al. (1987) *Cancer Res.* 47:4961-4966;
Shupnik et al. (1982) *J. Biol. Chem.* 257:12161-12164) and
G-292 (American Type Culture Collection, Bethesda, MD;

- 5 -

CRL 1423) were cultured in McCoy's 5a medium (GIBCO;
Grand Island, NY) supplemented with 10% (G-292) or 15%
(Saos-2) fetal calf serum (FCS; GIBCO), in a humidified,
5% CO_2, 37°C incubator. Cell proliferation was monitored

5 by ^{3}H-thymidine incorporation. The method is a slight
modification of the procedure described by Tatakis et al.
(Tatakis et al. (1989) *Biochem. Biophys. Res. Commun.*
164:119-127). Briefly, cells were seeded in 24-well flat
bottom polystyrene dishes (Corning; Corning, NY) (0.9x10^6

10 cells/ml; 0.5 ml of cell suspension/well). After a 24 h
incubation period in McCoy's 5a medium supplemented with
10% FCS, the cells were washed twice with McCoy's 5a
medium and supplemented with 1 mg/ml BSA. The cells were
then cultured in McCoy's 5a medium with BSA for 24 h. At

15 the end of this 24 h period, the medium was removed and
either McCoy's 5a medium with BSA or McCoy's 5a medium
with BSA and human alpha thrombin (specific activity ca.
4,000 U/mg protein; Sigma Chemical Co., St. Louis, MO) or
McCoy's 5a medium with FCS was placed in the wells (0.5

20 ml/well). PF4 (Calbiochem, San Diego, CA) was then added
to the wells (in all experiments PF4 was added last, and
at least 5 min after the addition of FCS or thrombin).
The cells were then incubated for another 24-48 h.
During the last three hours of this incubation period ^{3}H-

25 thymidine (1 μCi/ml; ICN, Irvine, CA) was added to the
medium. After the labeling period the cells were washed
once with McCoy's 5a medium and then extracted with TCA.
The acid-precipitable material was dissolved in KOH,
neutralized with HCl, and counted in Ecoscint A (National

30 Diagnostics; Manville, NJ) scintillation fluid.
        Referring to FIGS. 1A to 1C, PF4 inhibited
proliferation of G-292 cells cultured in medium
supplemented with 0.1% BSA (Fig. 1A), 2% FCS (Fig. 1B) or
0.1% BSA and 0.5 U/ml human alpha thrombin (Fig. 1C).

35 PF4 was effective in this system at doses between 20

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ng/ml and 2 μg/ml. In experiments with these cells, the
average maximum response of the cells to 2% FCS and 0.5
U/ml thrombin was ~600% and ~750% of basal thymidine
incorporation, respectively.

5   Referring to FIGS. 2A to 2C, PF4 inhibited
proliferation of Saos-2 cells cultured in medium
supplemented with 0.1% BSA (Fig. 2A), 2% FCS (Fig. 2B) or
0.1% BSA and 0.5 U/ml human alpha thrombin (Fig. 2C). In
experiments with these cells, the average maximum
10 response of the cells to 2% FCS and 1.0 U/ml thrombin was
~1100% and ~370% of basal thymidine incorporation,
respectively.

        G-292 cells appeared to be much more susceptible
to the action of PF4, compared to Saos-2 cells. A 100
15 ng/ml dose of PF4 caused a 75% inhibition of basal
proliferation in G-292 cells (Fig. 1A), while it had no
effect on Saos-2 cells (Fig. 2A). Thrombin-stimulated
cells (Fig. 1C and Fig. 2C) were the least affected by
the inhibitory action of PF4, with the Saos-2 cells again
20 being less susceptible. The degree of PF4-induced
inhibition of FCS-stimulated cells (Fig. 1B and Fig. 2B)
was close to the degree of inhibition under basal
conditions (Fig. 1A and Fig. 2A) for both cell lines.

        The doses at which PF4 effectively inhibited
25 proliferation of either cell line are lower than those
required for stimulation of elastase activity (Lonky et
al. (1978) *Biochem. Biophys. Res. Commun.* 85:1113-1118),
inhibition of collagenase (Hiti-Harper et al. (1978)
Science 199:991-992), or inhibition of endothelial cell
30 proliferation (Maione et al. (1990) *Science* 247:77-79;
Sharpe et al. (1990) *J. Natl. Cancer Inst.* 82:848-853),
and comparable to the doses required for reversal of
immunosuppression (Katz et al. (1992) *Int. Immunol.*
4:183-190) and stimulation of monocyte (Deuel et al.
35 (1981) *Proc. Natl. Acad. Sci (USA)* 78:4584-4587) or

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fibroblast chemotaxis (Senior et al. (1983) *J. Cell Biol.*
96:382-385). The PF4 doses effective against the
osteosarcoma cells fall in the high range of normal PF4
values in plasma (mean ± SD = 13.9 ± 6.1 ng/ml) and

5 within the PF4 levels that are theoretically possible at
sites of activation of the coagulation cascade and
release of platelet contents (Zucker et al. (1991) *Proc.*
Soc. Exp. Biol. Med. 198:693-702; Files et al. (1981)
Blood 58:607-618).

10   PF4 inhibited osteosarcoma cells grown either in
the absence of any stimulus or in the presence of two
differently acting stimulants, FCS and thrombin,
suggesting that PF4 has direct effects on intrinsic
mechanisms regulating the proliferation of osteosarcoma

15 cells. Without being bound to any particular theory, the
most likely mechanism of PF4 action would be the binding
to a specific membrane receptor.

        PF4 inhibited FCS-and thrombin-stimulated cells
even though it was added five minutes after the addition

20 of these agonists to the cells, suggesting that PF4
operates at a post-receptor stage of the stimulation
process. The time element obtains particular
significance when one considers that the thrombin-
elicited intracellular signals occur within the first

25 minute after addition of the enzyme to these cells
(Tatakis et al. (1989) *Biochem. Biophys. Res. Commun.*
164:119-127; Tatakis et al. (1991) *Biochem. Biophys. Res.*
Commun. 174:181-188). A separate set of experiments
demonstrated that PF4 inhibits thrombin-stimulated cells

30 to similar extent when it is added to the cells prior to
the addition of thrombin.

        A cytotoxicity assay demonstrated that the PF4-
induced inhibition of osteosarcoma cell proliferation was
not due to a cytotoxic effect of PF4 on these cells.

35 Cytotoxicity was assessed using an LDH release assay.

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Briefly, cytotoxicity (LDH release) assay kits, based on
a published procedure (Korzeniewski et al. (1983) *J.
Immunol. Methods* 64:313-320), were obtained from Oxford
Biomedical Research (Oxford, MI). For cytotoxicity
5   experiments cells were seeded in 96-well flat bottom
polystyrene dishes (0.25×10^6 cells/ml; 0.2 ml of cell
suspension/well). After 24 h growth period in McCoy's 5a
medium supplemented with 10% FCS, the cells were washed
twice with McCoy's supplemented with 1 mg/ml BSA. The
10 cells were then cultured in McCoy's with BSA for 24 h.
At the end of which the medium was removed and McCoy's
with 2% FCS was placed in the wells (0.2 ml/well) with or
without PF4 (six replicates/treatment group). The cells
were then incubated for another 24 h. During the last
15 three hours of this 24 h incubation period ^{3}H-thymidine
(1 μCi/ml) was added to half the wells of each treatment
group. The remaining three wells received lysing agent,
in order to determine maximum LDH release values for each
treatment group. After the 3 h period, a 100 μl aliquot
20 was removed from each of the 6 wells and used in the LDH
assay according to the manufacturer's instructions. The
wells that had received ^{3}H-thymidine were then treated as
above for proliferation.

These experiments demonstrated that PF4 doses that
25 caused significant inhibition of DNA synthesis had no
significant effect on LDH release by Saos-2 or G-292
cells (Table 1).

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TABLE 1: Effect of PF4 on osteosarcoma cell proliferation and viability

Treatment	^{3}H-thymidine incorporation (% of maximum)	Cell viability (% of maximum LDH release)
G-292 cells:		
2% FCS	100.0 ± 2.2	15.8 ± 3.4
FCS + PF4 2 μg/ml	8.3 ± 1.3[a]	17.7 ± 3.8
Saos-2 cells:		
2% FCS	100.0 ± 1.0	10.9 ± 0.6
FCS + PF4 2 μg/ml	68.4 ± 2.4[a]	8.8 ± 0.6

Results are the mean ±S.E. of the mean from two experiments, each performed in triplicates. [a]p<0.001 from FCS alone.

The fact that PF4 did not cause any cytotoxic effects on the osteosarcoma cells, as assessed by LDH release measurements, is consistent with the reported lack of cytotoxic effects of PF4 on endothelial cells (Maione et al. (1990) *Science* 247:77-79), and the lack of toxic effects when administered *in vivo* (Maione et al. (1991) *Cancer Res.* 51:2077-2083).

A separate set of experiments demonstrated that indomethacin did not effect PF4-induced inhibition of osteoblastic osteosarcoma cell proliferation, suggesting that this effect is independent of any PF4-elicited prostaglandin synthesis. Cell proliferation was measured as described above.

Referring to Fig. 3A, Saos-2 cells were cultured with 0.1% BSA, 200 ng/ml PF4, 1% FCS, or 1% FCS and 200 ng/ml PF4 in the presence of 1 μM indomethacin (hatched bars) or solvent only (ethanol, 0.1% final concentration; solid bars). For each treatment, proliferation is expressed as a percentage of the control level.

Referring to Fig. 3B, G-292 cells were cultured with 0.1% BSA (c), 200 ng/ml PF4, 0.5 U/ml human alpha thrombin (Thr), or 0.5 U/ml human alpha thrombin (Thr)

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and 200 ng/ml PF4 in the presence of 1 μM indomethacin
(hatched bars) or solvent only (ethanol, 0.1% final
concentration; solid bars). For each treatment,
proliferation is expressed as a percentage of the control
5   level.

Prostaglandins have direct effects on
osteosarcoma cell proliferation (Ren et al. (1992)
Calcif. Tissue Int. 50:372-377) and cytokines modulate
osteoblastic cell growth through simulation of endogenous
10 PG production (Tatakis et al. (1989) *Biochem. Biophys.
Res. Commun.* 162:435-440). Indomethacin, a prostaglandin
synthesis inhibitor, significantly inhibits the PF4-
induced reversal of immunosuppression (Zucker et al.
(1991) *Proc. Soc. Exp. Biol. Med.* 198:693-702; Katz et
15 al. (1992) *Int. Immunol.* 4:183-190), suggesting that this
immunoregulatory activity of PF4 requires prostaglandin
production. However, indomethacin failed to alter the
PF4-induced inhibition of osteoblast-like osteosarcoma
cell growth. This suggests that the PF4 effect on
20 osteosarcoma cells is prostaglandin-independent.

Naturally occurring or recombinant PF4 or
fragments thereof can be used in the method of the
invention. The production and cloning of PF4 have been
described previously (Poncz et al., *Blood* 69:219, 1987;
25 Cooke et al., *Circulation* 85:1102, 1992). These methods
may be used to produce PF4 for use in the method of the
invention. Methods for production of PF4 are also
described in PCT Application WO 85/04397 (Oncogen, Inc.).
Fragments of PF4 such as PF4 58-70 and PF4 47-70
30 (Rucinski et al., *Thromb. Haemost.* 63:493-498, 1990) and
monomeric low-affinity PF4 (LAPF4), which is 50%
homologous to PF4 and contains an α-helical C-terminus
(Mayo, *Biochem.* 30:925-934, 1990), may be useful in the
method of the invention. Other PF4 fragments potentially
35 useful in the method of the invention are described by

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Rucinski et al. (*Thrombosis and Haemostasis* 63:493, 1990)
and Maione et al. (*Science* 247:77, 1989).

Modified and mutant forms of PF4 may be useful in
the method of the invention. Such forms are described by
5 Maione et al. (U.S. Patent 5,086,164) and Maione (U.S.
Patent 5,112,946).

Various animal models are available for assessing
the effectiveness of PF4 in the method of the invention.
For example, canine models of osteosarcoma are commonly
10 used. Ovariectomized rats provided a model of
osteoporosis. Tumor necrosis factor or prostaglandins
can be injected into the calvarium to provide a model of
inflammation-induced resorption (e.g., as a model of
periodontal disease).

15 Use

In administering PF4, conventional pharmaceutical
or veterinary practice may be employed to provide
suitable formulations or compositions for administration
by any convenient means, for example, intravenous,
20 subcutaneous, intramuscular, intraventricular,
intracranial, intracapsular, intraspinal, intracisternal,
intraperitoneal, or oral administration.

PF4 may also be administered by surgical implants
which release formulations which include PF4.

25   Parenteral formulations may be in the form of
liquid solutions or suspensions; for oral administration,
formulations may be in the form of tablets or capsules.

Methods well known in the art for making
formulations are to be found in, for example,
30 "Remington's Pharmaceutical Sciences." Formulations for
parenteral administration may, for example, contain as
excipients sterile water or saline, polyalkylene glycols
such as polyethylene glycol, oils of vegetable origin, or
hydrogenated naphthalenes, biocompatible, biodegradable
35 lactide polymer, or polyoxyethylene-polyoxypropylene

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copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable
5 infusion systems, and liposomes.

PF4 can be used as the sole active agent, or can be used in combination with other active ingredients, e.g., other compounds which could regulate osteoblast or osteoclast function.

10 The concentration of PF4 for administration in the method of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, PF4 may be provided in an
15 aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall
20 health of the patient, the make up of the formulation, and the route of administration.

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What is claimed is:

1. Platelet factor 4 ("PF4") for use in inhibiting proliferation of osteoblasts in a mammal in need of such inhibition.

2. The use of platelet factor 4 ("PF4") for the
5 manufacture of a medicament for inhibiting proliferation of osteoblasts in a mammal in need of such inhibition.

3. The PF4 of claim 1 or 2 wherein said mammal is a human.

4. The PF4 of claim 1 or 2 wherein said human is
10 suffering from osteosarcoma.

5. The PF4 of claim 1 or 2 wherein said human is suffering from ossifying fibroma.

6. The PF4 of claim 1 or 2 wherein said human is suffering from osteoid osteoma.

15   7. The PF4 of claim 1 or 2 wherein said human is suffering from fibrous dysplasia.

8. The PF4 of claim 1 or 2 wherein said human is suffering from osteoporosis.

9. A method for inhibiting proliferation of
20 osteoblasts in a mammal in need of such inhibition comprising administering platelet factor 4 ("PF4").

10. The method of claim 9 wherein said mammal is a human suffering from osteosarcoma, ossifying fibroma, osteoid osteoma, fibrous dysplasia, or osteoporosis.

FIG. 1a

FIG. 1b

FIG. 1c
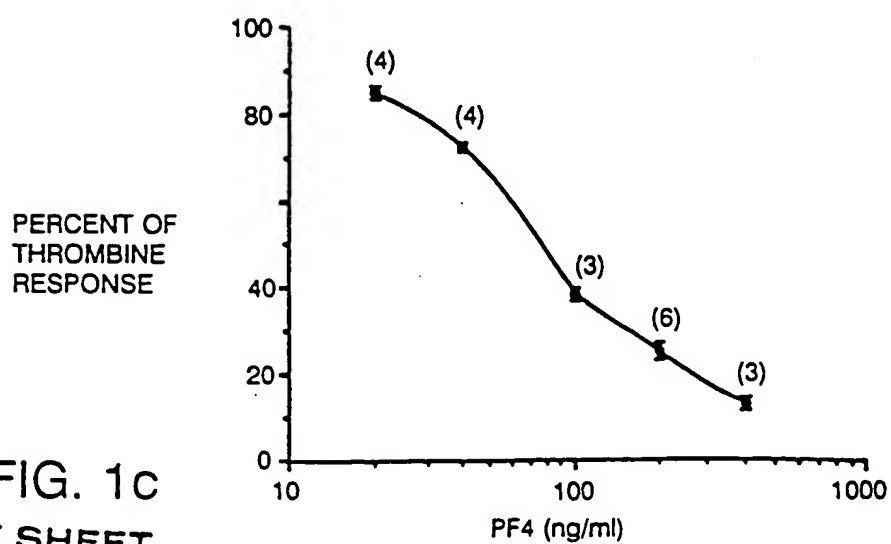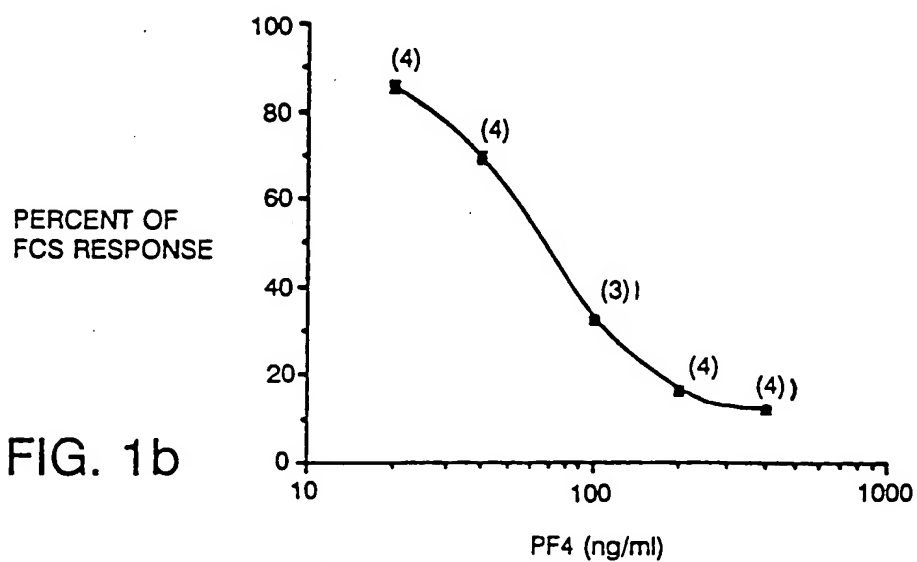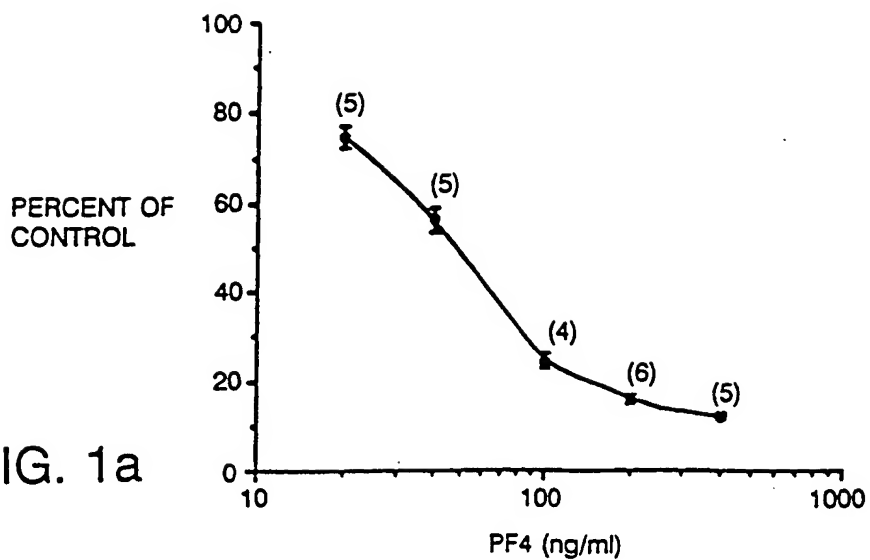
SUBSTITUTE SHEET

FIG. 2a

PERCENT OF
CONTROL

FIG. 2b
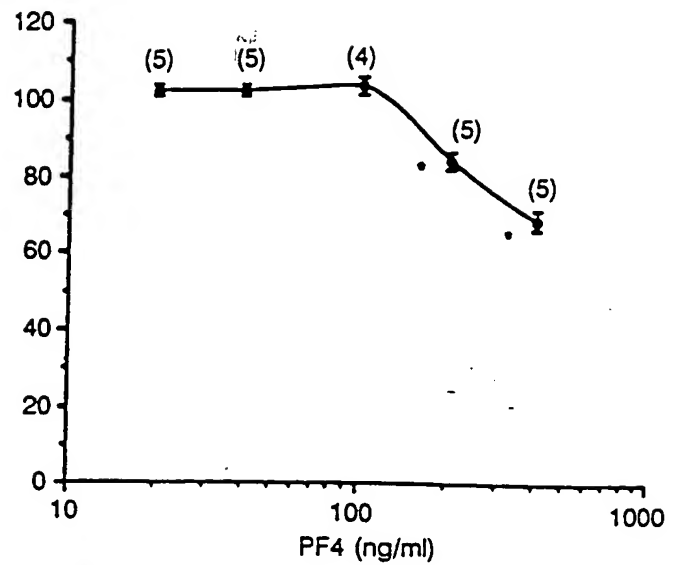
PERCENT OF
FCS RESPONSE

FIG. 2c
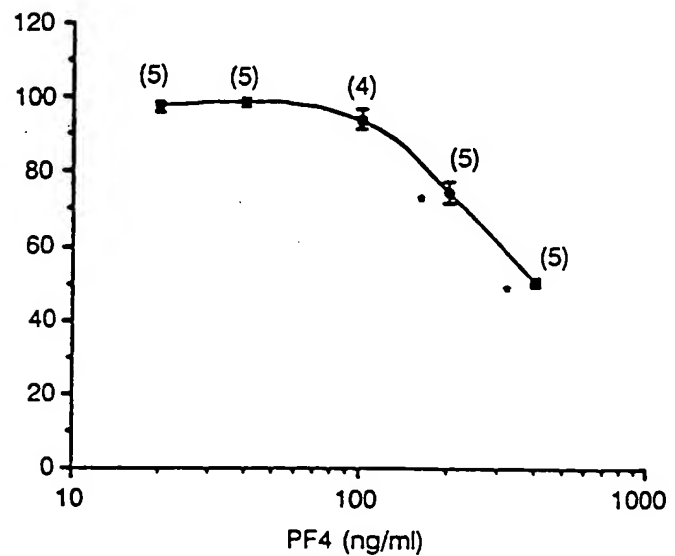
PERCENT OF
THROMBINE
RESPONSE
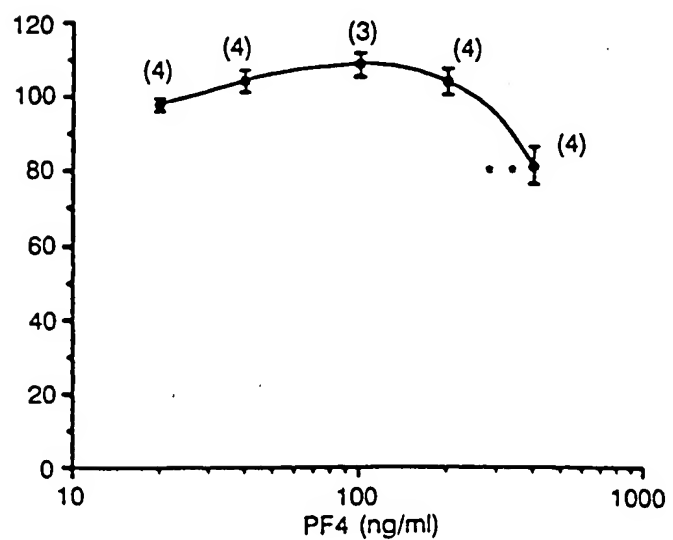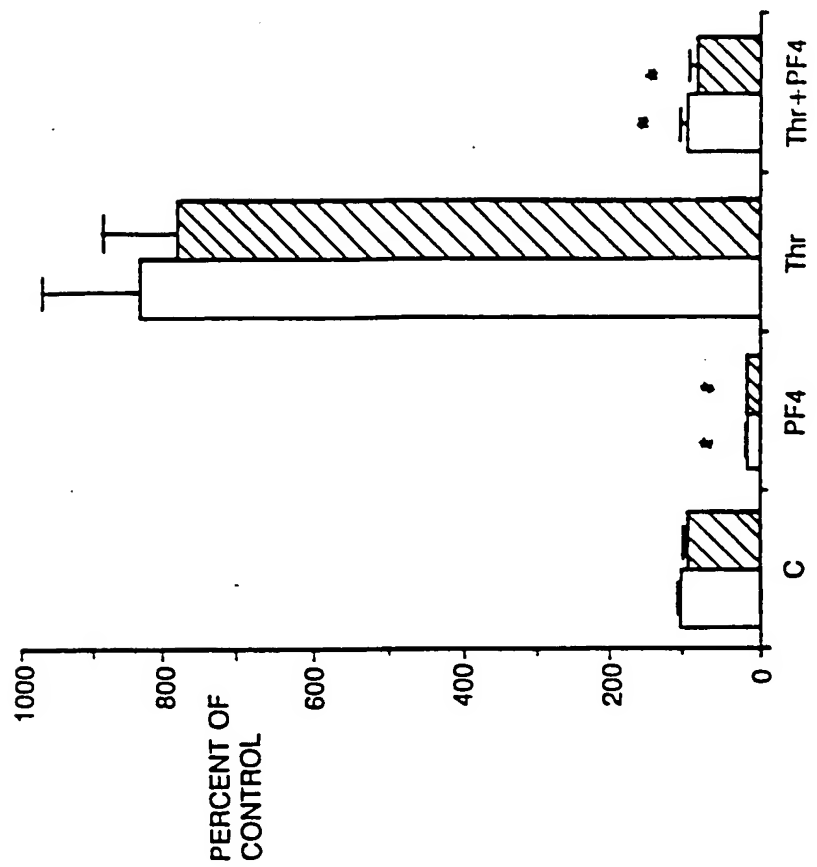
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FIG. 3b
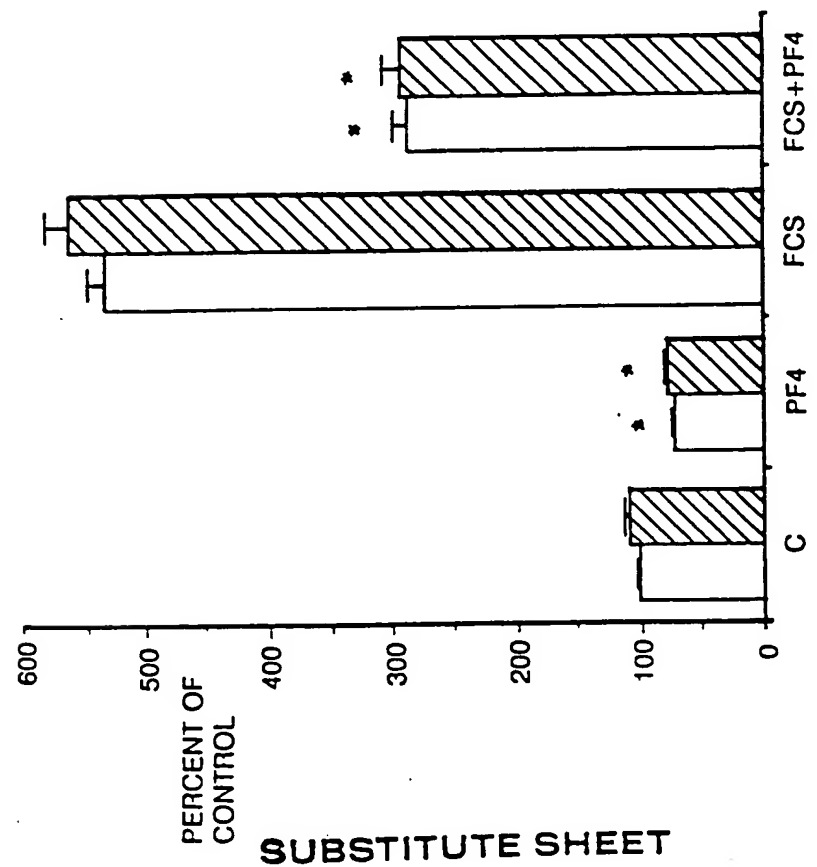
FIG. 3a

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A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00, 45/05
US CL :530/380, 824; 424/532, 85.2; 514/21

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/380, 824; 424/532, 85.2; 514/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

cas, bIOSIS, APS

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,086,164 (MAIONE ET AL.) 04 FEBRUARY 1992, see col. 3, lines 13-15.	1-10
Y	WO, A, 85/04397 (TWARDZIK) 10 OCTOBER 1985, see page 3, page 13, lines 10-15, page 14, lines 3-15.	1-10

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 October 1993	OCT 19 1993

Name and mailing address of the ISA/US	Authorized officer
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